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ISOLATION, CLONING AND EXPRESSION OF THE GENES FOR MICROBIAL POLYURETHANE DEGRADATION.

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Authors--First Report
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Project Scientist
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Abstract

New degradation tests are in progress to determine if polyurethane (Impranil®) and/or urethane (Sigma) can be used as sole carbon source. Fifteen different cultures have been inoculated into 10 ml polyurethane- and urethane-minimal salts solutions. Proteins have been extracted from a one liter culture of HAFB-2F with an ammonium sulfate precipitation procedure. 200 µl of a $(NH_4)_2SO_4$ treated solution was placed on a Pharmacia FPLC (Fast Protein Liquid Chromatography) system. Five fractions were collected from the column and will be assayed for polyurethane degrading activity. Isolation of DNA from HAFB-2F-Br (isolate) has begun. The DNA vector and fungal host, received from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center are cosmid pSV50 in an *E. coli* host, LM83, and *Neurospora crassa* wild type strain (74-OR23-1VA), FGSC #2489. The genomic library will be constructed in *E. coli* HB101. The fungal vector host will be used for expression of the genes from HAFB-2F-Br. While the DNA isolation procedure is being optimized, concurrent experiments will be done to optimize the transformation frequency of both the *E. coli* competent cells and the *Neurospora crassa* host using previously published procedures.

Identifiers/Open-Ended Terms
DNA/DNA libraries/16 S RNA sequence/
polyurethane paint/biodegradation/enzyme

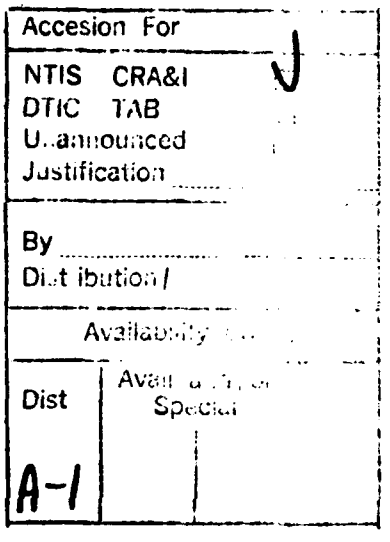
Availability
Defense Technical Information Center
Bldg. 5, Cameron Station
Alexandria, Virginia 22314

Security Class
Unclassified
Unlimited

FORWARD

This Technical Report covers work performed on Contract N00014-90-C-0182, entitled "Isolation, Cloning and Expression of the Genes for Microbial Polyurethane Degradation", technically from November 15, 1990 through February 15, 1991. This program is sponsored by the Office of Naval Research, 800 North Quincy Street, BCT #1, Arlington, Virginia 22217-5000. The Project Scientist is Captain Steve Snyder.

Mrs. Gail Bowers-Irons was both the Project Manager and Principal Investigator. Mr. Robert Pryor, Ms. Usha Charyulu and Dr. Ramesh Prakash, all U.S. citizens, were responsible for this quarter's experimentation.



Second Phase Experimental:

Urethane Growth

New degradation tests are in progress to determine if polyurethane (Impranil®) and/or urethane (Sigma) can be used as sole carbon source. Fifteen different cultures have been inoculated into 10 ml polyurethane- and urethane-minimal salts solutions. The 16 X 150 mm, Bellco stainless steel culture tube capped tubes have been placed in a tube rotator at a 30° angle. The rotator runs at 80 rpm and room temperature. The cultures include:

ATCC 53921	A TRA mixed paint culture
HAFB-2	A TRA bacterial paint culture
HAFB-2F	A TRA mixed paint culture
TF	A TRA plastic fungi isolate
PF	A TRA fungi isolate
DO	A TRA mixed culture
S1	A TRA mixed culture
S2	A TRA mixed culture
P1	A TRA mixed culture
P2	A TRA mixed culture
ATCC 35698	*Arthobacter globiformis*--*bacteria*
ATCC 11172	*Pseudomonas putida*--bacteria
ATCC 10196	*Aspergillus oryzae*--fungi
ATCC 9642	*Aspergillus niger*--fungi
ATCC 12668	*Trichoderma sp.*--fungi

The minimal salts medium is as follows:

72 Minimal

$(NH_4)_2SO_4$	1.000 gm/L
KH_2PO_4	5.000 gm/L
$MGSO_4 * 7H_2O$	0.100 gm/L
$FESO_4 * 7H_2O$	0.005 gm/L
Stock Salts Sol'n	1.000 ml/L
Carbon source of choice	1%

pH 7.0 w/NaOH before autoclaving.

Stock Salts Sol'n per 1 liter

MgO	10.75 gm
$CaCO_3$	2.00 gm
$FESO_4 * 7H_2O$	4.50 gm
$ZNSO_4 * 7H_2O$	1.44 gm
$MnSO_4 * 4H_2O$	1.12 gm
$CoSO_4 * 7H_2O$	0.28 gm
$CUSO_4 * 5H_2O$	0.25 gm
H_3BO_3	0.06 gm
HCl (conc.)	51.30 ml

The carbon sources were added to the above medium, 72 Minimal, after autoclaving. In first tests, 1.45 ml of aqueous polyurethane was added to 500 ml of medium. This converts to 0.2% of polyurethane material in solution. To 475 ml of 72 Minimal medium, 0.95 gm of urethane was added to make a 0.2% solution.

The above urethane and polyurethane media were also made into solid media with the addition of 5.0 gm Gel-Gro® (ICN Biochemicals) and 0.47 gm of $MgCl_2 * 6H_2O$. The media were then poured into 100 mm diameter petri dishes. The plates were also inoculated with the fifteen cultures.

These tests show positive growth on polyurethane with the TF, HAFB-2, PF and ATCC 9642 cultures. In the urethane tests, positive growth has been shown with the TF, HAFB-2, HAFB-2F, PF and ATCC 10196 cultures.

An isocyanate/urethane colorimetric test is being developed. The test is based on work by C. Hepburn in *Polyurethane Elastomers*. This test will use *p*-dimethylaminobenzaldehyde as reagent and will measure the rate and extent of degradation of the urethane.

Proteins

Proteins have been extracted from a one liter culture of HAFB-2F with an ammonium sulfate precipitation procedure. The culture was filtered through a Büchner funnel using a 15 cm Fisher Scientific P4 qualitative filter paper. 460.82 gm of $(NH_4)_2SO_4$ was added to the filtrate to produce an 80% salt. The solution was then spun at high speed (25,000 rpm) for 30 minutes. The supernatant was discarded and the pellets were resuspended in 10 ml of TES buffer (15 mM Tris-pH 8, 5 mM EDTA, 100 mM NaCl). To bring the concentration to 40%, 2.31 gm of $(NH_4)_2SO_4$ was added. This solution was centrifuged as before and the pellet discarded. The supernatant was taken to 80% with the addition of 2.64 gm $(NH_4)_2SO_4$. This solution was centrifuged. The pellet was saved and resuspended in 10 ml TES buffer.

200 µl of this solution was placed on a Pharmacia FPLC (Fast Protein Liquid Chromatography) system. The FPLC used a HR 10/30 column with Superose resin and TES elution buffer. The flow rate was 1.0 ml/min. UV wavelength detection was placed at 280 nm and refractive index was 225 nm. Chart paper speed was 0.5 cm/min. Figure 1 shows this chart. Five fractions were collected from the column. These fractions were run on a 15% SDS-Page gel at 70 volts overnight. The visible resolution of the gel was poor due to the low protein concentration used. The gel will be run again using a larger sample. The five fractions will also be assayed

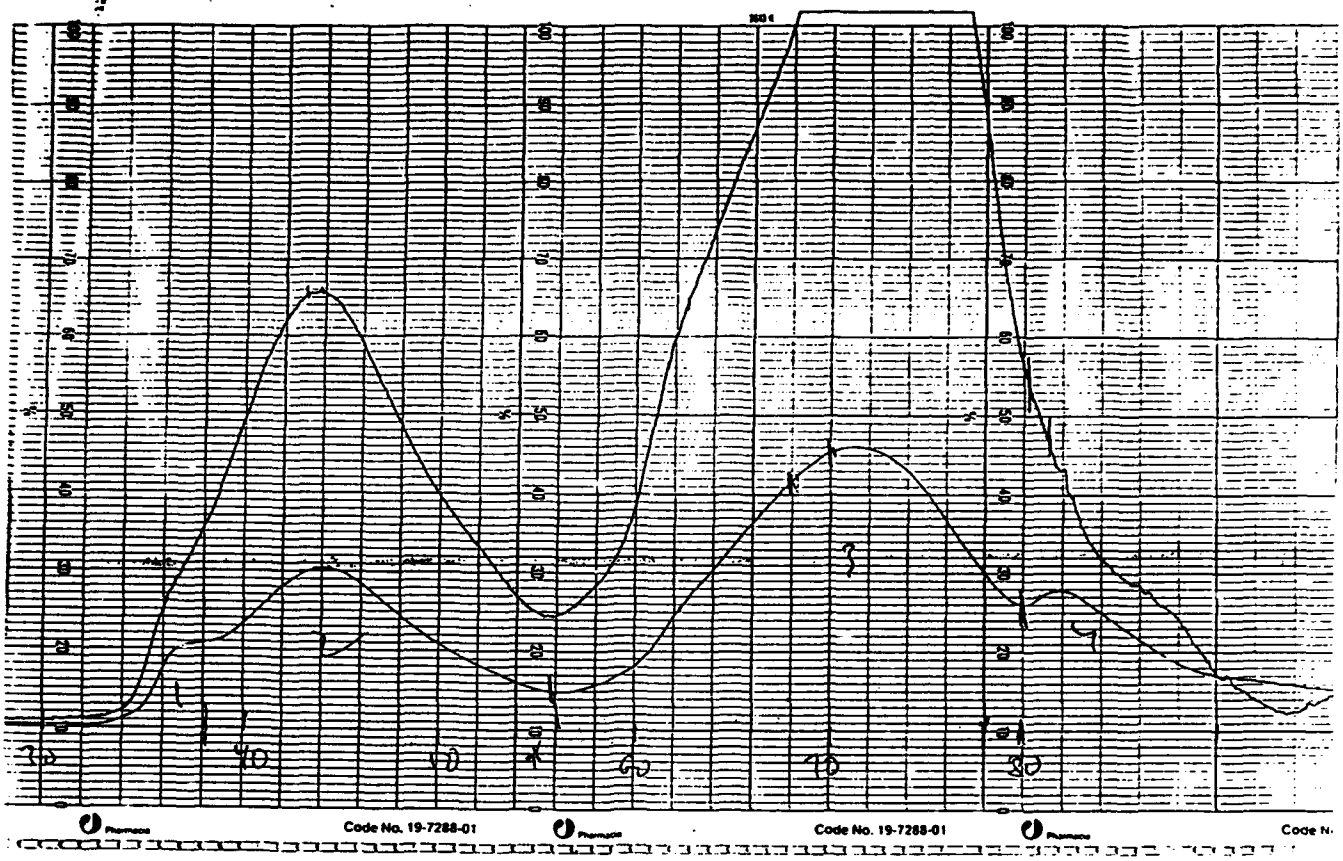


Figure 1 FPLC column detector plot
Top trace: Refractive Index 225 nm
Bottom tract: UV abs @ 280 nm
Time in minutes along bottom

DNA

Isolation of DNA from HAFB-2F-Br (isolate) has begun. A one liter culture is being grown in NB medium to provide approximately one gram of dried mycelium. The mycelium will be ground under liquid nitrogen after drying and weighing. The ground material will then be incubated at 65°C for 10 minutes in extraction buffer (50mM Tris-pH 8.0, 100 mM EDTA, 250 mM NaCl, 1% SDS). The DNA will then be extracted with a 3:1 mixture of phenol:chloroform and centrifuged at 10,000 rpm for 60 minutes. After centrifuging, the mixture will be extracted with chloroform and then treated with RNase solution (20 mg/ml in Tris-pH 7.5, 15 mM NaCl) at 37°C for 30 minutes. The mixture will again be extracted with chloroform and the DNA precipitated with 0.6 volumes of isopropanol. The yield will be then determined by the optical density at 260 nm. Approximately 100 µg of DNA will be needed for construction of the genomic library. The purity of the DNA will also be check by measuring the optical density at 280 nm, the absorption band for proteins. Pure DNA has an O.D. 260/280 ratio of 1.5-2.0. Depending on the yield and purity obtained from the above procedure, some modifications to the procedure may be necessary.

A partial digestion will then be done on 1 µg of DNA using *Sau* 3A restriction enzyme over 10, 20 and 30 minute intervals. These partial digestions will be run on a gel with undigested DNA using λ *Hin* dIII digest as a marker. The digestion time that yields 5-10kb will then be used for construction of the genomic library.

The DNA vector and fungal host have been received from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center. They are cosmid pSV50 (Figure 2) in an *E. coli* host, LM83, and *Neurospora crassa* wild type strain (74-OR23-1VA), FGSC #2489. The genomic library will be constructed in *E. coli* HB101. The fungal vector host will be used for expression of the genes from HAFB-2F-Br. The cosmid in its *E. coli* host and the *E. coli* host for the library construction will be grown in LB medium. *Neurospora crassa* will be grown in Vogel's medium.

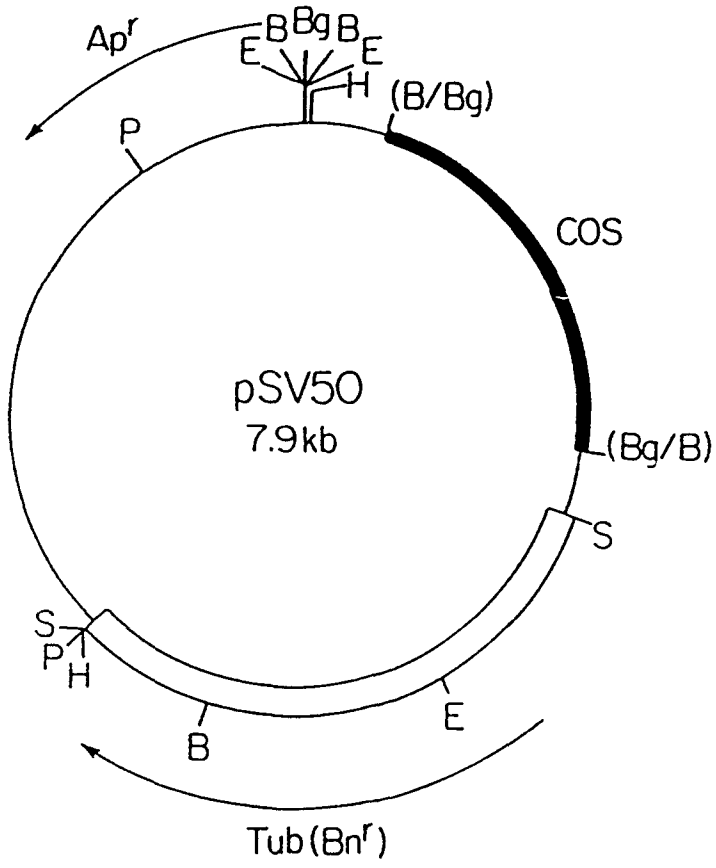


Figure 2 Cosmid pSV50
Restriction enzyme sites:
B, *Bam* HI;Bg, *Bgl* II;E, *Eco* RI;H, *Hin* dIII;P, *Pst* I;S, *Sal* I
Tub(Bn^r: Benomyl Resistance
Ap^r: Ampicillin Resistance[1]

The cosmid DNA will be purified from the host and digested with the restriction enzyme *Bgl* II and then treated with alkaline phosphatase to keep the fragments from re-ligating. The fragments will be run on a low melting agarose gel to separate them. The largest of the three fragments will be extracted from the gel and used as the vector. The procedure removes the cos site and a small piece of unnecessary DNA from the cosmid, which without the cos site, is now a plasmid. The cos site is not needed because we are not going to do in vitro packaging. The vector was chosen for its ability to transform both *E. coli* and *Neurospora crassa*.

While the DNA isolation procedure is being optimized, concurrent experiments will be done to optimize the transformation frequency of both the *E. coli* competent cells and the *Neurospora crassa* host using previously published procedures.[1-4]

NB Medium

Beef Extract	3 gm/L
Peptone	5 gm/L
pH 6.8	

LB Medium

Tryptone	10 gm/L
Yeast Extract	5 gm/L
NaCl	5 gm/L
pH 7.5 with NaOH	

Vogel's Medium

Sodium Citrate	2.0 gm/L
NH_4NO_3	1.0 gm/L
KH_2PO_4	1.0 gm/L
$MgSO_4*7H_2O$	0.5 gm/L
NaCl	0.1 gm/L
$CaCl_2$	0.1 gm/L
Biotin	2-5 mg/L
Stock Salts Sol'n	1.0 ml/L
Sucrose	15.0 gm/l

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